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Structure of the intermediate species of the photoreaction cycle of bacteriorhodopsin

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Abstract. Bacteriorhodopsin, a chromoprotein having retinal as chromophore is linked to protein opsin through a protonated Schiff base, and undergoes a cyclic photoreaction on light absorption involving a number of intermediate species. The conformational preferences of the protonated snd deprotonated Schiff bases of all-*trans* and 13-*cis* retinal with *n*-pentylamine as model for lysine have been investigated using molecular orbital PCILO (Perturbative Configuration Interaction of Localised Orbitals) method. The theoretical calculations reveal that there is an intrinsic differential flexibility between the protonated and deprotonated Schiff bases irrespective of the retinal isomers. The deprotonation of the Schiff base enhances the flexibility of the chromophore in the molecule. Based upon this information and that there are no major changes in the conformation of the protein opsin, two models for the photoreaction cycle involving intermediates of different structures have been proposed. Of these, experimental evidences seem to favour model 1 in which deprotonation and no *trans-cis* isomerisation accompanies the primary photochemical event. In other words, the intermediate species of the photoreaction cycle of bacteriorhodopsin have all-*trans* retinal as the chromophore with varying degree of non-covalent interaction with the protein opsin.

Keywords. Retinal; all-*trans* retinal; 13-*cis* retinal; protonated Schiff base; bacteriorhodopsin.

1. Introduction

Bacteriorhodopsin (bR), a chromo-protein containing aldehyde of vitamin A as the chromophore, is a membrane protein which occurs in the cellular purple membrane of halobacteria (Stoeckenius and Rowen 1967; Oesterhelt and Stoeckenius 1971; Stoeckenius 1976, 1980). It is structurally similar to the visual pigment rhodopsin (Wald 1968; Honig 1978) but its biological role is quite different. It is the only other photosynthetic system besides the chlorophylls known to exist in nature. In chlorophylls a series of oxidation-reduction processes convert the absorbed light energy into an electrochemical proton gradient across the membrane which contains the pigment, while in bacteriorhodopsin the light energy is directly converted into an electrochemical proton gradient across the membrane. The energy stored in the electrochemical proton gradient is then used by the cells

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to synthesize ATP and to drive other transport processes. Thus, bacteriorhodopsin functions as a light driven proton pump.

Bacteriorhodopsin is a relatively small protein consisting of 247 amino acid residues and has a molecular weight of around 27,000 daltons. The primary sequence of this protein has recently been determined (Khorana *et al* 1979; Ovchinnikov *et al* 1979) and it has been established that the chromophore retinal is attached to the ϵ-amino group of lysine which is 41 residue distant from the N-terminal amino acid of the molecule. The hydrophobic amino acids constitute about 62% of the polypeptide chain which is largely buried in the membrane. The electron micrographs and electron diffraction (Unwin and Henderson 1975; Henderson 1975; Henderson and Unwin 1975) have shown that bacteriorhodopsin molecule contains seven helical segments about 40 Å long and 10 Å apart extending nearly across the membrane which is about 45 Å in width and almost perpendicular to the membrane plane.

Bacteriorhodopsin shows a strong absorbance band around 568 nm which is about 200 nm red shifted from the band ($\sim$ 370 nm) normally observed for a retinal Schiff base. This large red shift has been demonstrated to be partially due to the protonated form of the Schiff base (Morton and Pitt 1958; Suzuki and Kito 1972; Kropf and Hubbard 1958) and partially due to environmental effects arising from the non-covalent interactions with the polypeptide chain of the protein. The actual mechanism of this significant red shift ($\sim$ 200 nm), however, still remains to be established. If bacteriorhodopsin is kept in the dark, its absorbance maximum shifts to 558 nm with a concomitant decrease of intensity by about 15%. This form is called dark-adapted bacteriorhodopsin (bR^{DA}_{558}). Exposure to moderate light intensities restores the light adapted form (bR^{LA}_{568}) within few seconds. Chemical extraction of retinal with organic solvents from bR^{LA}_{568} yields nearly exclusively all-*trans* retinal while bR^{DA}_{558} yields equal amounts of the 13-*cis* and all-*trans* isomers (Stoeckenius 1980). It has been suggested that differences in interactions with protein stabilize the two isomers of the retinal in the light and dark adapted forms.

Bacteriorhodopsin undergoes a cyclic photoreaction the kinetics of which have been studied by low temperature absorption spectroscopy and flash photolytic method (Kung *et al* 1975; Shapiro *et al* 1978; Applebury *et al* 1978; Hurley *et al* 1978). The pronounced absorbance changes of the chromophore during the photoreaction cycles has been utilized to characterize the cycle. It is believed that *trans-cis* isomerization is the primary photochemical event which is followed by deprotonation and reprotonation of the Schiff base. The complete photoreaction takes about 10 m sec and during each cycle two protons have been suggested to be translocated (Becher and Ebrey 1977). These calculations are based on the quantum efficiencies for cycling $\approx 0{\cdot}3$ (Goldschmidt *et al* 1977; Becher and Ebrey 1977) and proton pumping $\approx 0{\cdot}6$ (pH $< 7{\cdot}0$) (Hartmann *et al* 1977; Bogomolni *et al* 1980; Govindjee *et al* 1980), respectively. Although flash photolysis (Kung *et al* 1975), low temperature spectroscopy (Lozier *et al* 1975) and resonance Raman studies (Terner *et al* 1977, 1979; Aton *et al* 1977) have been utilized to work out the details of the photoreaction and the structure of photocycle intermediates, the inferences drawn from these studies are still ambiguous. Not only the structure of intermediate species even their number is still

questioned (Stoeckanius 1980). If there is a *trans-cis* isomerisation accompanying the photochemical event, then at some other stage in the photoreaction cycle, a *cis-trans* isomerisation will have to be invoked to explain the existence of all-*trans* retinal as chromophore in bR_{568}^{LA}. Theoretical calculations (Waddell and Hopkins 1977; Kakitani and Kakitani 1975; Salem and Bruckman 1975; Warshel 1976; Suzuki *et al* 1974) on retinal and its Schiff bases with amines predict high potential barriers for *cis-trans*/*trans-cis* isomerisation. The possibilty of the existence of barrierless torsion potential energy around C13–C14 double bond has been suggested (Honig 1978). The high *cis-trans* quantum yields in the triplet manifold observed for 11-*cis* retinal and its Schiff base (Menger and Kliger 1976) have also been suggested as a possible pathway for lowering this potential barrier for *cis-trans* interconversion.

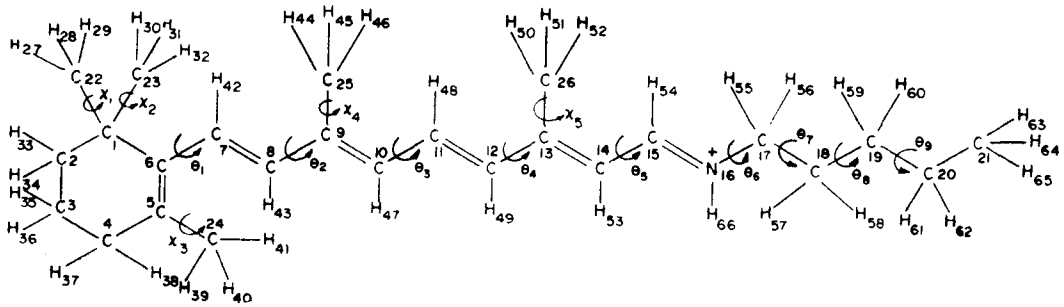
In this paper we report the results of our theoretical calculations on protonated and deprotonated Schiff bases of all-*trans* and 13-*cis* retinal with *n*-pentyl amine as a model for Schiff base of chromophore in bR, as these are the only two isomers which have been detected in the extraction of the chromophore. The aim is to determine the minimum energy conformers for the protonated and deprotonated Schiff bases and their relevance to the intermediate species of the photoreaction cycle of bacteriorhodopsin.

2. Method of calculation

The method utilized for the determination of preferred conformers of Schiff bases of all-*trans* and 13-*cis* retinals is the PCILO method (Pullman and Saran 1976). The geometry of retinal molecule adopted for the computation is the same as discussed in the preceding paper (Dhingra and Saran 1981) while that of the *n*-pentyl amine was constructed using standard bond lengths and bond angles.

Figure 1 shows the schematic diagram of the all-*trans* retinal Schiff base with *n*-pentyl amine and the various torsional angles are defined as:

χ_1 = C2–C1–C22–H27, χ_2 = C2–C1–C23–H30, χ_3 = C4–C5–C24–H39, χ_4 = C8–C9–C25–H44, χ_5 = C12–C13–C26–H50; θ_1 = C5–C6–C7–C8, θ_2 = C7–C8–C9–C10, θ_3 = C9–C10–C11–C12, θ_4 = C11–C12–C13–C14, θ_5 = C13–C14–C15–N16,



PROTONATED SCHIFF BASE

Figure 1. Schematic diagram of protonated Schiff base of all-*trans* retinal.

θ_6 = C15–N16–C17–C18, θ_7 = N16–C17–C18–C19, θ_8 = C17–C18–C19–C20, and θ_9 = C18–C19–C20–C21, with the *cis*-planar arrangement of the terminal bonds being taken as torsion angle equal to zero. The clockwise rotation of the distant bond relative to the near bond is taken as the positive value of the torsion angle. Conformational energy maps have been constructed as a function of two torsion angles by keeping the other torsion angles fixed in their preferred values. The computations have been carried out in 30° intervals of the torsion angle and the presentation of the results on the maps has been limited to the 5 kcal/mole isoenergy curves.

3. Results

First, the $(\theta_7$–$\theta_6)$ conformational energy map has been constructed for deprotonated Schiff base of all-*trans* retinal by fixing $\theta_8 = \theta_9 = 180°$ and the protons of the terminal methyl group in the staggered conformation. The conformation of the retinal part is the same as obtained by Dhingra and Saran (1981) with $\theta_5 = 180°$. The results of this map have been utilized to construct the $(\theta_8 - \theta_7)$ and $(\theta_9 - \theta_8)$ conformational energy maps to obtain the preferred values of θ_6, θ_7, θ_8 and θ_9. These values have then been, adopted for the construction of the $(\theta_6 - \theta_5)$ conformational energy map.

3.1 *Conformation of Schiff base of all-trans retinal*

3.1a *Deprotonated Schiff base* : Figure 2 shows the conformational energy map for deprotonated Schiff base of all-*trans* retinal and it can be seen that there are two global minima, one at $\theta_7 = 60°$ and $\theta_6 = 120°$ and the other at $\theta_7 = 300°$ and $\theta_6 = 240°$ having exactly the same energy. In addition to these, there are four local minima within 0·5 kcal/mole higher isoenergy curve: two at $\theta_7 = 180°$ and 300° associated with $\theta_6 = 120°$ and the other two at $\theta_7 = 60°$ and 180° and associated with $\theta_6 = 240°$. Of these, the local minima associated with $\theta_7 = 60°$ and 300° are about 0·2 kcal/mole higher than the global ones while the remaining two local minima associated with $\theta_7 = 180°$ are about 0·35 kcal/mole higher in energy. We have, thus, two preferred values of $\theta_6 = 120°$ and 240° and three values of $\theta_7 = 60°$, 180° and 300°.

For the construction of the next map, i.e., the $(\theta_8 - \theta_7)$ conformational energy map, we have adopted $\theta_6 = 120°$ and $\theta_9 = 180°$ and this map is shown in figure 3. It can be seen that the global minimum occurs at $\theta_8 = \theta_7 = 300°$. The energy of this global minimum is about 0·1 kcal/mole lower than that of the global minimum of the map shown in figure 2. There are four local minima within 0·5 kcal/mole isoenergy curve and they occur at $(\theta_8, \theta_7) = (60°, 60°)$, (180°, 60°), (180°, 180°) and (180°, 300°). In addition there are three low energy regions (upto 1 kcal/mole) at $(\theta_8, \theta_7) = (60°, 180°)$, (60°, 300°) and (180°, 300°).

Figure 4 shows the conformational energy constructed as a function of θ_9 and θ_8 by fixing $\theta_6 = 120°$ and $\theta_7 = 300°$. This map shows two global minima, one at $\theta_9 = \theta_8 = 300°$ and the other at $\theta_9 = 180°$ and $\theta_8 = 300°$. Of these, the global minimum at $\theta_9 = \theta_8 = 300°$ is about 0·1 kcal/mole lower in energy than that at $\theta_9 = 180°$ and $\theta_8 = 300°$. Thus, there is only one preferred value for θ_8 (= 300°) while both 300° and 180° are preferred for θ_9.

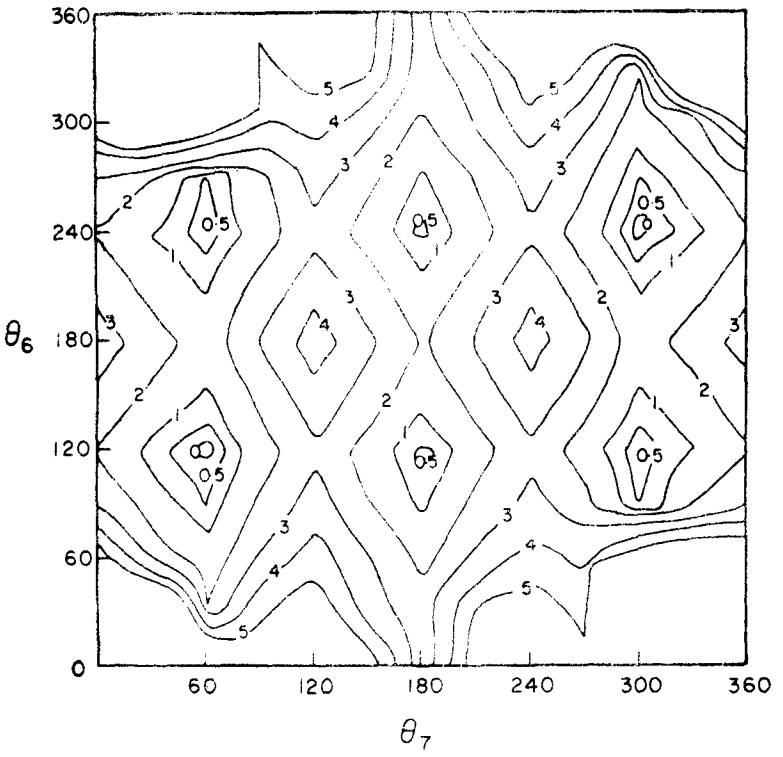


Figure 2. The $(\theta_7 - \theta_6)$ conformational energy map for deprotonated Schiff base of all-*trans* retinal constructed with $\theta_8 = \theta_9 = 180°$. Isoenergy curves in kcal/mole with the global minimum taken as energy zero.

The conformational energy map as a function of torsion angles θ_6 and θ_5 has been constructed by fixing $\theta_7 = \theta_8 = \theta_9 = 300°$. One can see (figure 5) that there are six global minima corresponding $(\theta_6, \theta_5) = (120°, 30°)$, $(120°, 180°)$, $(120°, 330°)$, $(240°, 30°)$, $(240°, 180°)$ and $(240°, 330°)$. This map essentially predicts greater flexibility of deprotonated Schiff base of all-*trans* retinal for θ_5 than for θ_6. All the six global minima are enclosed within 2 kcal/mole isoenergy curves and the barrier height between these minima is of the order of only 1 kcal/mole.

3.1b. *Protonated Schiff base* : To construct the $(\theta_6 - \theta_5)$ conformational energy map for protonated Schiff base of all-*trans* retinal (figure 6) the torsion angles θ_7, θ_8 and θ_9 have been fixed as in figure 5 (i.e. $\theta_7 = \theta_8 = \theta_9 = 300°$). One can see that this map is quite restricted as compared to the deprotonated case (figure 5) and most of two-dimensional (θ_6, θ_5) hyperspace is forbidden. There is only one global minimum (as compared to six in the deprotonated Schiff base) at $\theta_6 = 120°$ and $\theta_5 = 180°$. There are two low energy regions about 2 kcal/mole higher in energy at the same value of $\theta_5 = 180°$ and $\theta_6 = 240°$ and $300°$. This map clearly reveals that the protonated Schiff base of all-*trans* retinal has limited flexibility as compared to the deprotonated Schiff base.

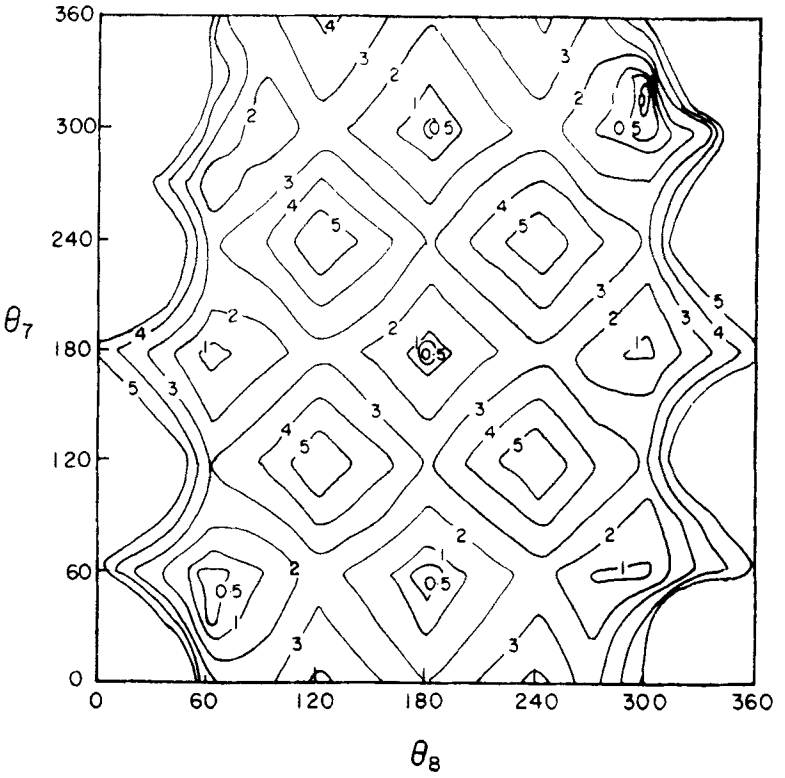


Figure 3. The $(\theta_8 - \theta_7)$ conformational energy map for deprotonated Schiff base of all-*trans* retinal constructed with $\theta_6 = 120°$, $\theta_9 = 180°$. Isoenergy curves in kcal/mole with the global minimum taken as energy zero.

3.2 *Conformation of Schiff base of* 13-*cis retinal*

3.2a. *Deprotonated Schiff base* : Similar to the Schiff base of all-*trans*, we have first constructed the $(\theta_6 - \theta_5)$ conformational energy for deprotonated Schiff base of 13-*cis* retinal. This map, which has been constructed with $\theta_7 = \theta_8 = \theta_9 = 300°$ is presented in Figure 7. There are three global minima in this case as compared to six in the all-*trans* retinal case (figure 5) and these occur at the same value of $\theta_6 = 120°$ and $\theta_5 = 150°$, 210° and 330° and they are all encompassed by a single 0·5 kcal/mole isoenergy curve. In addition to these, there are three local minima within 0·5 kcal/mole isoenergy curves at $\theta_5 = 30°$, 150° and 210° associated with $\theta_6 = 240°$. The energy of these local minima is exactly 0·4 kcal/mole above the global minimum. Thus, in this case of deprotonated Schiff base of 13-*cis* retinal, we have six regions of low energy ($\sim$ 0·4 kcal/mole). A comparison with deprotonated Schiff base of all-*trans* (figure 5) will reveal that positions of some of the low energy regions in figure 6 are slightly altered. The major difference between the two maps is that the torsion angle θ_6 is more restricted in the case of Schiff base of 13-*cis* retinal as compared to that in Schiff base of all-*trans* retinal.

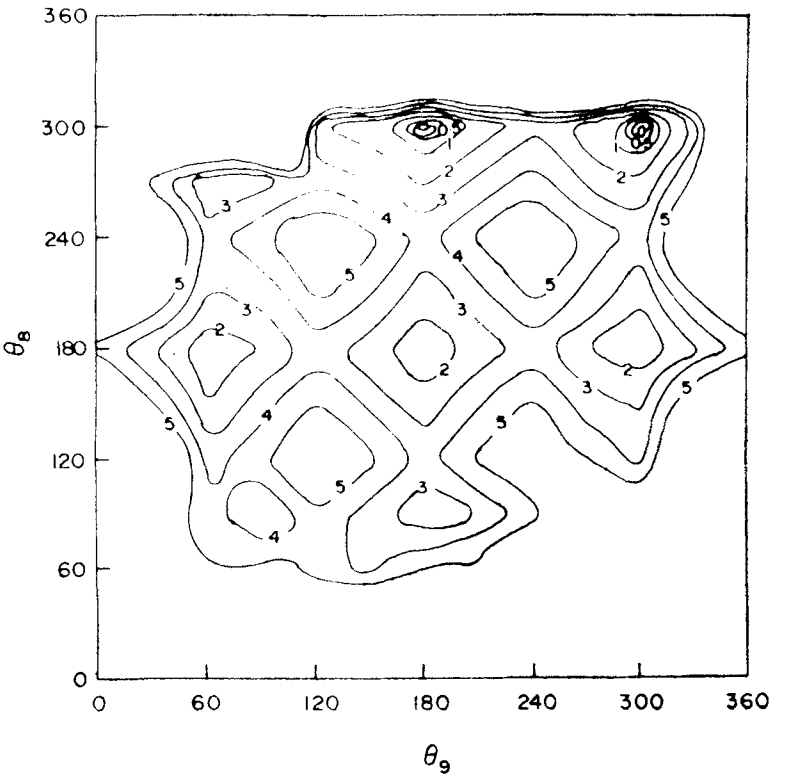


Figure 4. The $(\theta_9 - \theta_8)$ conformational energy map for deprotonated Schiff base of all-*trans* retinal constructed with $\theta_6 = 120°$, $\theta_7 = 300°$. Isoenergy curve in kcal/mole with the global minimum taken as energy zero.

3.2b *Protonated Schiff base* : Figure 8 shows the conformational energy map as a function of θ_6 and θ_5 for protonated Schiff base of 13-*cis* retinal. This map is very similar to that for the protonated Schiff base of all-*trans* retinal. The global minimum occurs at $\theta_6 = 120°$ and $\theta_5 = 180°$ with two low energy regions about 2 kcal/mole higher in energy at $\theta_6 = 240°$ and 300° associated with $\theta_5 = 180°$. A comparison between the maps for the protonated Schiff base of all-*trans*, and 13-*cis* reveals that the conformational flexibility for the torsion angles θ_6 and θ_5 are almost similar and thus independent of the isomeric form of the retinal.

4. Discussion

It is now well established that light adapted bacteriorhodopsin (bR^{LA}_{568}) which has all-*trans* retinal chromophore, undergoes a photoreaction cycle (figure 9) involving a number of intermediate species after absorbing a photon (Stoeckenius 1980). An understanding of the structure and kinetics of these species is fundamental to its functioning as a proton pump. The existing experimental data on bacteriorhodopsin indicate that there is no evidence of major conformational change in the protein opsin. It is, therefore, believed that the observed intermediate

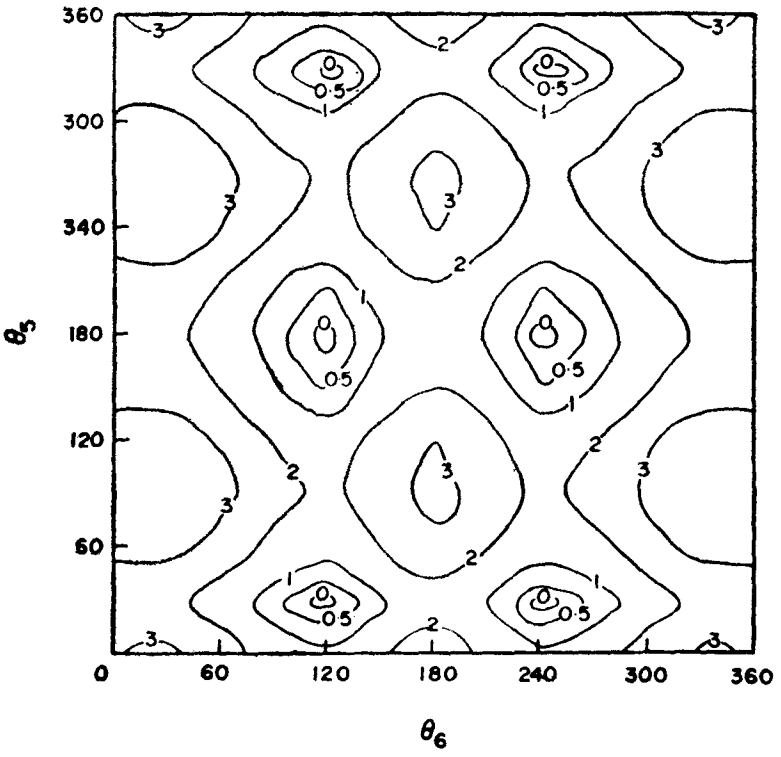


Figure 5. The $(\theta_6 - \theta_5)$ conformational energy map for deprotonated Schiff base of all-*trans* retinal constructed with $\theta_7 = \theta_8 = \theta_9 = 300°$. Isoenergy curves in kcal/mole with the global minimum taken as energy zero.

species are probably associated with the structural changes in the chromophore. The regulation of λ_{max} is governed by the conformational changes in the chromophore in conjunction with interactions with protein. In order to delineate the structure of various intermediate species, it is pertinent to know the stage at which deprotonation takes place and whether photochemical event is accompanied by *trans-cis* isomerisation or not. In analogy with rhodopsin, the visual pigment of the eye, it is believed that the primary photochemical event in bacteriorhodopsin is *trans-cis* isomerisation (Hubbard *et al* 1966). The resonance Raman spectroscopy (Aton *et al* 1977; Marcus and Lewis 1978) of one of the relatively long lived intermediates M_{412} has indicated that this intermediate is probably deprotonated Schiff base of 13-*cis* retinal and hence supports the *trans-cis* isomerisation hypothesis. This observation also suggests that deprotonation must have occurred during the conversion of intermediates formed before M_{412}, i.e., $L_{550} \rightarrow M_{412}$ or $K_{590} \rightarrow L_{550}$ or the photochemical isomerisation is accompanied with deprotonation. In order to resolve these points, there has been a considerable amount of experimental efforts and speculation on the structure K_{590} species. The time-resolved resonance Raman data (Terner *et al* 1977, 1979) indicate that the Schiff base of K_{590} is protonated while the low temperature data and isotope effect

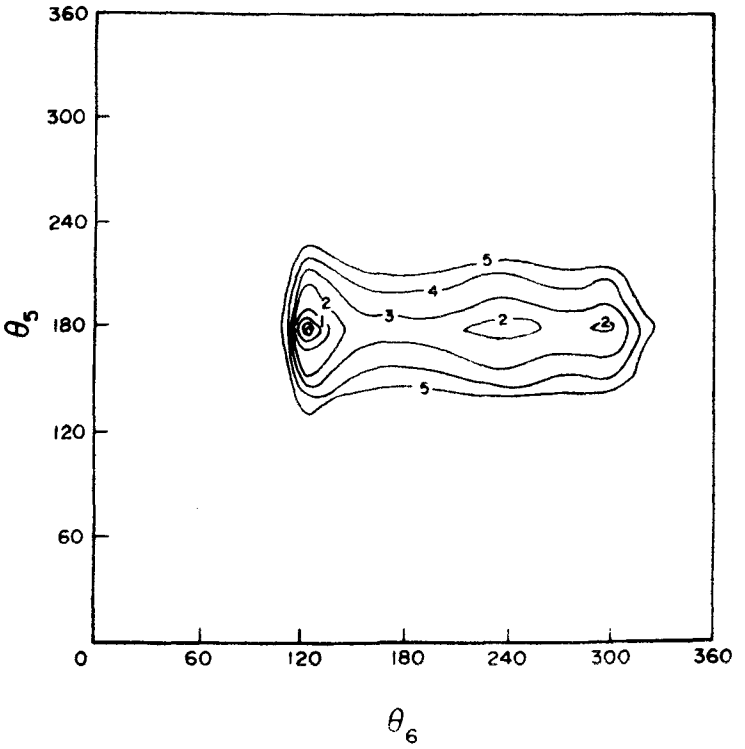


Figure 6. The $(\theta_6 - \theta_5)$ conformational energy map for protonated Schiff base of all-*trans* retinal constructed with $\theta_7 = \theta_8 = \theta_9 = 300°$. Isoenergy curves in kcal/mole with the global minimum taken as energy zero.

in D_2O (Applebury *et al* 1978) strongly suggest that a proton is transferred. Whether this is a Schiff base proton or some other proton is still controversial.

A comparison of the conformational behaviour of protonated and deprotonated Schiff bases of all-*trans* and 13-*cis* retinals presented earlier, reveals that protonation of the Schiff base makes the chromophore-pentylamine segment of the molecule relatively more rigid. This rigidity imparted by protonation is independent of the isomeric forms of the retinal. However, the present calculations indicate (table 1) that the protonated Schiff base of all-*trans* retinal is relatively more stable ($\sim$ 1·37 kcal/mole) than the corresponding Schiff base of 13-*cis* retinal. Similarly the deprotonated Schiff base of all-*trans* retinal is relatively more stable ($\sim$ 1·39 kcal/mole) than the corresponding Schiff base of the 13-*cis* retinal. This difference in relative flexibility around the two bonds, i.e., C14–C15 (θ_5) and N16–C17 (θ_6) which are next to the site where protonation or deprotonation occurs might play a key role in the observation of structurally different intermediates of the photoreaction cycle and regulation of λ_{max}.

In the light of our present theoretical calculations which predict subtle differences in the intrinsic flexibilities of protonated and deprotonated Schiff bases, we propose two models for the structure of intermediates of the photoreaction cycle.

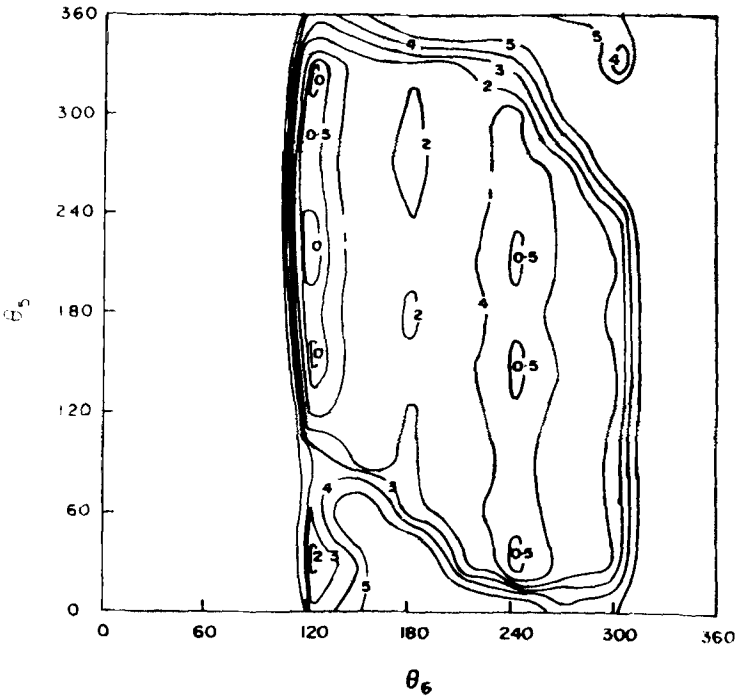


Figure 7. The $(\theta_6 - \theta_5)$ conformational energy map for deprotonated Schiff base of 13-*cis* retinal constructed with $\theta_7 = \theta_9 = \theta_9 = 300^\circ$. Isoenergy curves in kcal/mole with the global minimum taken as energy zero.

Table 1. Energies of protonated and deprotonated Schiff base of all-*trans* and 13-*cis* reitnal.

Retinal isomer	Protonated Schiff base		Deprotonated Schiff base	
	Absolute energy	Relative* energy	Absolute energy	Relative* energy
All-*trans*	− 138550·01	0·0	− 138194·51	0·0
13-*cis*	− 138548·64	1·37	− 138193·12	1·39

*Relative to all-*traus*. All energy values are in kcal/mole.

4.1 *Model* 1

This model is based on the assumption that there is no *trans-cis* isomerisation of the retinal and the primary photochemical event is the translocation of proton of the Schiff base. The justification for this assumption is provided by the recent

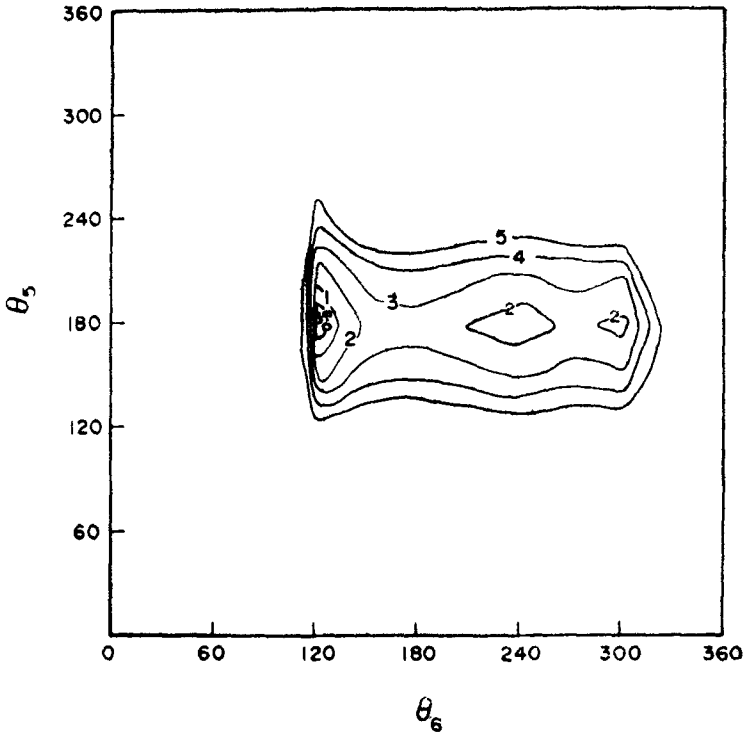


Figure 8. The $(\theta_6 - \theta_5)$ conformational energy map for protonated Schiff base of 13-*cis* retinal constructed with $\theta_7 = \theta_8 = \theta_9 = 300^\circ$ Isoenergy curves in kcal/mole with the global minimum taken as energy zero.

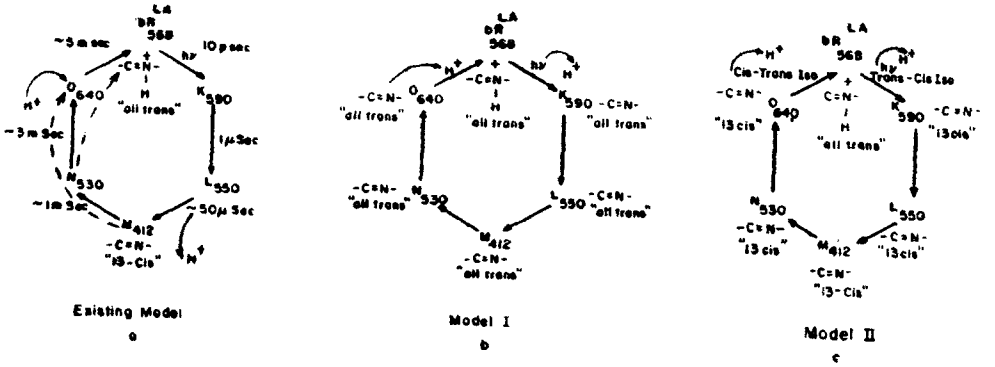


Figure 9. Photoreaction cycle of bacteriorhodopsin ; (a) existing model (Stoeckenius 1980), (b) our proposed model 1. (c) our proposed model 2.

picosecond spectroscopy of rhodopsin (Peters *et al* 1977) and bacteriorhodopsin (Shapiro *et al* 1978) in low temperature glasses which indicate that 6–10 picosecond rise time of K_{590} (i.e., the first intermediate of the photoreaction cycle) is too short for the photochemical *trans-cis* isomerisation but it is consistent with translocation of proton. Hence, the primary photochemical event could be either translocation of a proton of the Schiff base or an excited state of bR. Since this

specie has long life time (~ 300 picosecond) it cannot be an excited state of bR. As our theoretical calculations indicate that protonation controls the flexibility of the molecule, we believe that the primary photochemical event could be the translocation of a proton of the Schiff base. Once the proton is translocated, the inherent flexibility associated with the deprotonated Schiff base of all-*trans* would lead to a number of minimum energy conformers. Figure 5 shows that there are six minimum energy conformers possible for the deprotonated Schiff base of all-*trans* retinal. Each of these conformers would be stabilized by interaction with the protein opsin, and this differential interaction would regulate the λ_{max} of the chromophore. Although it is known that protonation brings about red shift of about 50–60 nm, the major fraction of red shift of bacteriorhodopsin is believed to come from protein–chromophore interaction. It is, therefore, argued that untill it is proved that the protein-chromophore interaction is mediated and regulated through the Schiff base proton, it is quite likely that the deprotonated Schiff base with enhanced protein-chromophore or chromophore-lipid interactions could be responsible for the regulation of λ_{max}. Our proposed model 1 based on the above deductions and the existing model are shown in figure 9. The main feature of our model is that one does not have to invoke the *cis-trans* isomerisation during the dark period of the photoreaction cycle. This is one of the unanswered questions of the existing model (figure 9) in which the primary photochemical process is believed to be *trans-cis* isomerisation. Even if the *cis-trans* isomerisation takes place, the question remains to be answered is what is the source of energy for this process which requires energy of the order of about 25 kcal/mole. As pointed earlier, the short rise time (~ 6–10 pico sec) of K_{590} intermediate is consistent with translocation of proton rather than the *trans-cis* isomerisation. These two arguments augment the validity of the model 1 which we propose here on the basis of theoretical calculations. An additional experimental support for model 1 comes from the study of chromophore mobility in bacteriorhodopsin. The significant rotational rate constant which has been found to be 20 sec^{-1} at room temperature, is too high to be attributed to the rotation of the protein molecule and thus suggests an internal conformational change of the chromophore (Sherman and Caplan 1977).

4.2 *Model 2*

In this model the absorption of a photon has dual role of *trans-cis* isomerisation and proton translocation. In other words, all the intermediates of the photoreaction cycle in model 2 will have 13-*cis* retinal as chromophore and the Schiff base will be deprotonated. The protonation and *cis-trans* isomerisation will take place during the transformation of O_{640} specie to bR^{LA}_{568}. However, it is very difficult to explain the source of energy of the order of about 25 kcal/mole which is needed for isomerisation of *cis* form to *trnans* form. This inexplicable feature of model 2 lends further credence in favour of model 1.

5. Conclusions

Similar models can be proposed for the photoreaction cycle of the dark adapted bacteriorhodopsin which is believed to have 13-*cis* retinal as a chromophore. In

this case, once again, the primary photochemical event will be translocation of proton only. It can be stated that model 1 in which deprotonation and no *trans-cis* isomerisation accompanies the primary photochemical event is the most plausible model based on the experiemental evidences and the present theoretical study.

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